

(FILE 'HOME' ENTERED AT 14:31:56 ON 17 AUG 2005)

L1 FILE 'EMBASE, BIOSIS, MEDLINE, CAPLUS' ENTERED AT 14:32:10 ON 17 AUG 2005
2 S CHLORINE AND MDR1A

L2 FILE 'EMBASE, BIOSIS, CAPLUS, MEDLINE' ENTERED AT 14:43:52 ON 17 AUG 2005
14 S INFLAMMATORY BOWEL DISEASE AND CHLORINE
L3 13 DUP REM L2 (1 DUPLICATE REMOVED)
L4 2 S MDR1A AND CHLORINE



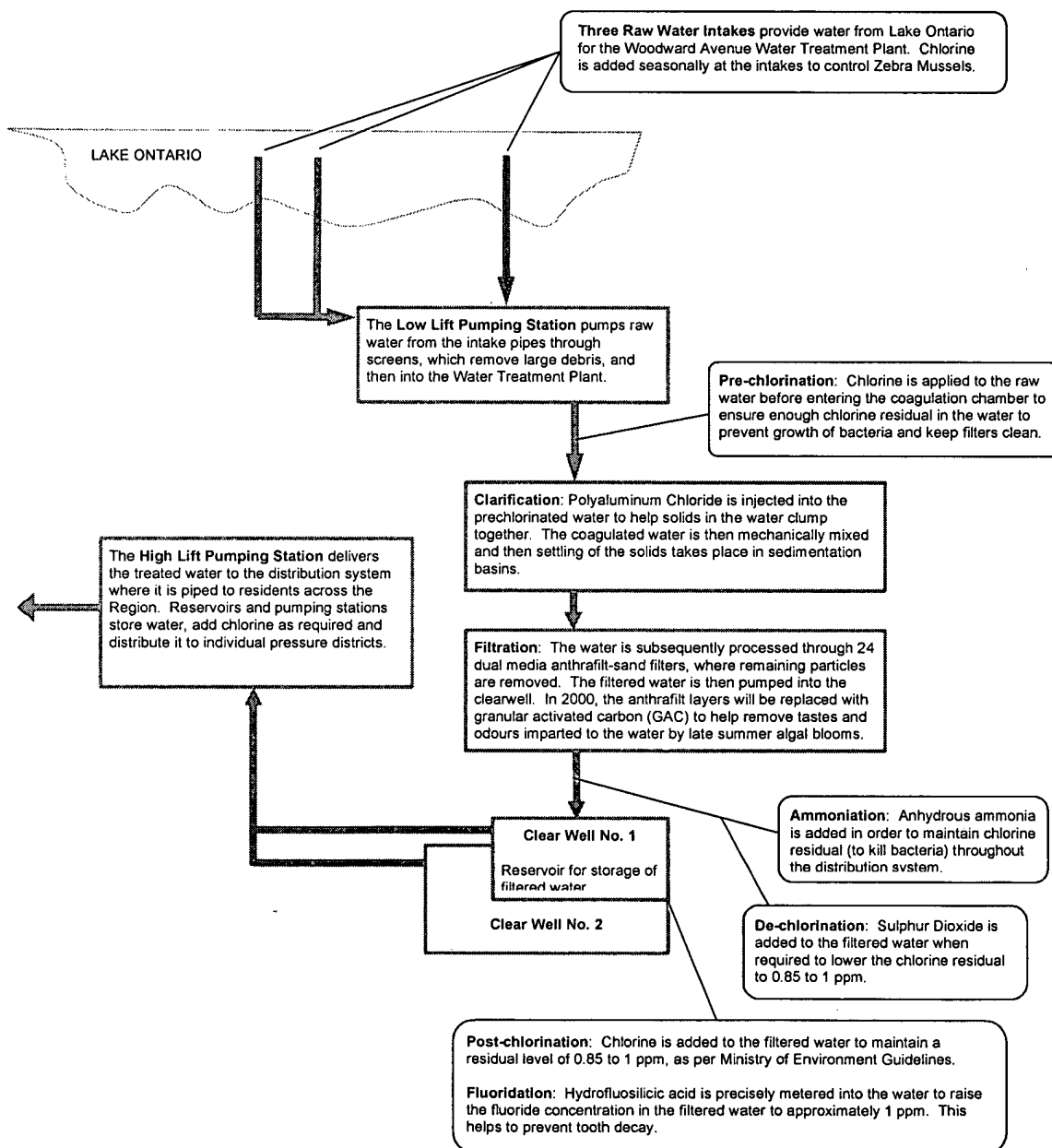
City of Hamilton and Region of Hamilton-Wentworth

1999 DRINKING WATER QUALITY REPORT



Welcome to our first edition of the Drinking Water Quality Report! This report provides information about the quality of drinking water The Region of Hamilton-Wentworth delivers to its customers, where the water comes from, and how it is treated. These reports will be presented each year and will serve to keep you informed on your drinking water and how The Region of Hamilton-Wentworth is continuing to work hard to provide you with safe, reliable and high-quality drinking water. We are very pleased with the 1999 results and we hope you can take a few minutes to read through the report. We welcome any questions or comments you may have and contacts are listed at the end of the report.

Woodward Avenue Water Treatment Plant



BEST AVAILABLE COPY

Samples are analyzed by the Regional Environmental Laboratory (REL) located at the Woodward Avenue Water Treatment Plant (WTP). The REL is accredited by the Standards Council of Canada on recommendation by the Canadian Association for Environmental Analytical Laboratories for tests listed in their scope of testing. The accreditation is based on ISO Guide 25. The REL is managed by The Region of Hamilton-Wentworth.



Raw water samples are collected at the Low Lift Pumping Station and tested for ammonia, chloride, chlorine (free and total), conductivity, fluoride, pH and turbidity on a rotating schedule; three days one week and two days the next.

Treated water samples are collected at the High Lift Pumping Station at the Woodward Avenue WTP and tested for alkalinity, aluminum, ammonia, chlorine (free and total), conductivity, fluoride, hardness, nitrate, nitrite, pH and turbidity three times per day and on the same rotating schedule as the raw water. Treated water is also tested on a quarterly basis for metals and trihalomethanes. Trihalomethanes are disinfection by-products which are produced when chlorine reacts with naturally occurring organics in the water. Drinking water is also sampled for bacteria and chlorine residual on a regular basis both at the treatment plant and at over fifty locations within the distribution system.

Hamilton-Wentworth Regional Well Supply

There are four areas in the Regional Municipality of Hamilton-Wentworth that use ground water as their water source: Freeleton (2 wells), Carlisle (4 wells), Greensville (1 well), and Lynden (1 well). The wells are inspected by staff three times per week, during which time the chlorine residuals, turbidities and flows are checked. The wells are equipped with on-line continuous chlorine residual analyzers (where chlorination is practiced).

The REL provide all the physical, chemical, organic and inorganic collection/testing/analysis for the wells and distribution system. The community wells are tested weekly for bacteria and chlorine; quarterly for metals and trihalomethanes; and colour, pH and turbidity are tested at some of the wells as required by the Ministry of Environment. Nitrate samples are taken at the well once per month in Greensville and pesticide samples are taken quarterly at the well in Lynden as these are considered agricultural areas.

Drinking Water Surveillance Program samples are collected for submission to the Ontario Ministry of the Environment two times per year from the plant, low-lift wet well, and two locations within the distribution system. The Region of Hamilton-Wentworth's drinking water consistently meets all Ontario Drinking Water Objectives.

Listed below are eight health related chemical/physical or microbiological parameters which are subject to Ontario Drinking Water Objectives which were detected in the Region of Hamilton-Wentworth's treated water during 1999. Several other health related parameters were tested for but were not detected, and therefore, are not listed in the table.

Glossary of Terms

Maximum Acceptable Concentration (MAC) - The highest level of a contaminant that is allowed in drinking water, as defined by the Ontario Drinking Water Objectives.

Parts Per Million (ppm) - Equivalent to milligrams per litre. One part per million is comparable to one penny in \$10,000.

Nephelometric Turbidity Unit (NTU) - A unit of measure for turbidity in a water sample.

Parts Per Billion (ppb) - Equivalent to micrograms per litre. One part per billion is comparable to one penny in \$1,000,000.

Measured at Woodward Avenue Water Treatment Plant				
PARAMETER (Measuring Unit)	Highest Level Allowed (MAC)	Highest Level Detected	Range Detected	Description and Origin of Substance
Barium (ppm)	1	0.025	0.019-0.025	A naturally occurring mineral found in most soils.
Fluoride (ppm)	1.5	1.38	0.21-1.38	Naturally occurring mineral; also added to drinking water to promote dental health.
Nitrate (ppm)	10	0.58	0.00-0.58	Presence due to runoff from fertilizer use; leaching from septic tanks, sewage; erosion of natural deposits.
Trihalomethanes (ppb)	100	24.0	10.9-24.0	By-products of drinking water chlorination.
Turbidity (NTU)	1	1.89 (2 of 545 samples >1)	0.02-1.89	Turbidity is a measurement of the cloudiness of water. It may be caused by inorganic soil particles or fragments of organic matter that can interfere with treatment.
Measured in Distribution System at Customer's Tap				
Lead (ppb)	10	5.20	0.13-5.20	Lead occurs naturally in soils, but its presence in drinking water is almost entirely due to corrosion of private household plumbing systems. (last monitored in 1998-97 by the Drinking Water Surveillance Program)
Trihalomethanes (ppb)	100	29.2	11.2-29.2	By-products of drinking water chlorination.
Escherichia coli (number of positive samples)	0	1 (1 of 2189 samples)	no range	Present in fecal matter and prevalent in sewage. It is a good indicator of recent fecal pollution.
Total Coliform Bacteria (number of positive samples)	2 consecutive positive samples not permitted	1 (1 of 2190 samples)	no range	Naturally occurring micro-organisms that are one indication of treatment effectiveness.
Measured in the Communal System (Wells)				
Nitrate (ppm)	10	7.38	4.98-7.38	Presence due to runoff from fertilizer use; leaching from septic tanks, sewage; erosion of natural deposits.
Trihalomethanes (ppb)	100	30.3	<0.8-30.3	By-products of drinking water chlorination.
Escherichia coli (number of positive samples)	0	4 (4 of 720 samples)	no range	Present in fecal matter and prevalent in sewage. It is a good indicator of recent fecal pollution.

HOW TO CONTACT US: City of Hamilton/Regional Municipality of Hamilton-Wentworth
55 John Street North, 6th Floor
Hamilton, Ontario L8R 3M8

For inquiries regarding low water pressure, unusual taste or colour of water contact:
Administrative Services, Water Distribution Section – 546-4426

For inquiries regarding quality of water contact:
Water Quality Section – 546-2140

Recent Innovations in Tissue-Specific Gene Modifications in the Mouse

Yasuhide Furuta and Richard R. Behringer*

Annotating the functions of individual genes in *in vivo* contexts has become the primary task of mouse genetics in the postgenome era. In addition to conventional approaches using transgenic technologies and gene targeting, the recent development of conditional gene modification techniques has opened novel opportunities for elucidating gene function at the level of the whole mouse to individual tissues or cell types. Tissue-specific gene modifications in the mouse have been made possible using site-specific DNA recombinases and conditional alleles. Recent innovations in this basic technology have facilitated new types of experiments, revealing novel insights into mammalian embryology. In this review, we focus on these recent innovations and new technical issues that impact the success of these conditional gene modification approaches. **Birth Defects Research (Part C) 75:43–57, 2005.**

© 2005 Wiley-Liss, Inc.

INTRODUCTION

The last decade in forward genetics in the mouse has witnessed another major advancement, owing to the successful development of conditional modifications of the mouse genome that complement conventional transgenic mouse and knockout (KO) approaches. Conditional gene modifications utilize site-specific recombinase technologies based on phage-derived Cre-loxP or Flp-floxed (Flp-FRT) systems in the mouse genome (Branda and Dymecki, 2004). Most significantly, these techniques have been used extensively to inactivate gene function in tissue-specific manners. Simply put, mice that express a recombinase in a tissue-specific manner are combined with mice carrying conditional alleles to create conditional KO

(CKO) mice. CKO approaches can overcome inherent problems found in conventional KO mouse models; i.e., if the gene of interest is essential for survival of embryos or animals, studies of gene function in a desired cell type, tissue type, or developmental time point are often precluded. Thus, using the myriad of available conditional alleles (e.g., discussed in a special issue of *Genesis*, vol. 32, issue 2, 2002, "Conditional Alleles in Mice," http://www.wiley.com/legacy/products/subject/life/anatomy/genesis_mice.html), CKO approaches have uncovered novel *in vivo* roles of genes in tissue-specific contexts in otherwise normal animals. More recently, tissue-specific gene modification technologies have evolved further to disrupt gene function in specific tissues at specific times. In

this review, we examine some of the most recent modifications and concerns of tissue-specific knockout technologies in the mouse. These new aspects have begun to reveal new insights into tissue-specific gene function.

RECOMBINASE REPORTER MICE

Important genetic tools that are required for recombinase-based tissue-specific gene modification systems are reporter mouse strains that indicate the *in vivo* activities and consequences of recombinases. Important basic features incorporated into recombinase reporter transgenic mice are the following two components. First, the reporter transgene, before recombinase-mediated recombination, is silent due to the presence of a DNA sequence, a so-called "STOP" cassette, upstream of the reporter gene that blocks reporter expression. This STOP cassette, many times a selectable marker for drug selection containing multiple polyadenylation (polyA) signals, is flanked by loxP or FRT sites. Second, the reporter transgene is designed to come under the control of a regulatory element that is ideally active in all cell types throughout embryonic and postnatal development. When such a reporter transgene allele is acted upon by the ap-

Yasuhide Furuta is from the Department of Biochemistry and Molecular Biology, University of Texas M.D. Anderson Cancer Center, and the Program in Genes and Development, Graduate School of Biomedical Sciences, University of Texas-Houston, Health Sciences Center and M.D. Anderson Cancer Center, Houston, Texas. **Richard R. Behringer** is from the Department of Molecular Genetics, University of Texas M.D. Anderson Cancer Center, and the Program in Genes and Development, Graduate School of Biomedical Sciences, University of Texas-Houston, Health Sciences Center and M.D. Anderson Cancer Center, Houston, Texas. Grant sponsor: National Institutes of Health (NIH); Grant numbers: EY13128 (to Y.F.), HD30284 (to R.R.B.); Grant sponsor: American Heart Association; Grant number: 035509Y (to Y.F.).

*Correspondence to: Richard R. Behringer, University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030. E-mail: rrb@mdanderson.org

Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/bdrc.20036

propriate recombinase, excision of the STOP cassette will occur, allowing expression of the reporter gene. Because the excision event activating the reporter modifies genomic DNA, the recombined allele is stably inherited by progeny cells. Reporter gene expression persists regardless of recombinase gene expression in the subsequent proliferation and differentiation of the cellular descendants. These features are also essential components that enable in vivo cell fate mapping in mice (Branda and Dymecki, 2004).

Currently, there are many useful recombinase reporter mouse strains available that employ various types of reporter genes (Lobe et al., 1999; Mao et al., 1999, 2001; Soriano, 1999; Kawamoto et al., 2000; Novak et al., 2000; Awatramani et al., 2001; Srinivas et al., 2001; Vintersten et al., 2004). The availability of several different reporter gene products allows choices of unique advantage, depending on the needs of a particular experiment. Prototype recombinase reporter alleles were developed using *lacZ* or human placental alkaline phosphatase (AP) reporters that allow visualization of recombined allele-carrying cells in situ using relatively easy histochemical methods on whole mount or sectioned material. More recently, new recombinase reporter alleles have been developed using green fluorescent protein (GFP) and other fluorescent protein color variants (e.g., yellow, YFP; cyan, CFP; red, RFP) (Novak et al., 2000; Mao et al., 2001; Srinivas et al., 2001; Vintersten et al., 2004).

The Z/AP Cre reporter mouse strain developed by Lobe et al. (1999) added another feature for recombinase reporter alleles. Prior to Cre-mediated recombination, the Z/AP allele expresses *lacZ* ubiquitously directed by a CAGG regulatory element (Niwa et al., 1991), but the AP reporter is silent. After Cre-mediated excision, the floxed *lacZ* gene is deleted and the AP reporter becomes expressed. A similar strategy was used to create the enhanced GFP (Z/EG) Cre reporter mouse strain (Novak et al., 2000). Z/AP and Z/EG were generated by random integra-

tion of CAGG driven recombinase reporters in embryonic stem (ES) cells. Thus, this strategy allows one to preselect ES cell clones that express *lacZ* in a ubiquitous pattern, and therefore also the second reporter after Cre-mediated recombination, prior to generating mice. In addition, the dual reporter system allows one to document both loss of one reporter and activation of the Cre-dependent reporter. A dual-recombinase-responsive reporter strain has also been generated (R26-FLAP), which allows visualization of cells that have undergone two recombination events along their developmental history when combined with two different Cre- and Flp-expressing alleles (Awatramani et al., 2003). Thus, reporter expression is only found in the overlap in the spatial and/or temporal pattern of expression of the two different recombinase transgenes.

The basic principles of these recombinase reporter transgenic strains have also been used to conditionally overexpress or ectopically express other genes by replacing the second reporter gene with a cDNA of interest. The Z/AP strategy has also been employed to create Z/(your gene of interest) mice, preselecting for the potential of "your gene of interest" to be expressed in any cell type after Cre-mediated recombination (Ding et al., 2002). This recombinase-mediated transgene expression system can be used to overcome problems of potential early lethality of founders using conventional transgenic approaches. Conceptually, dual-recombinase-responsive transgenes can also be developed that will allow the expression of introduced genes in more defined cell types in which the presence and activity of two different recombinase transgenes intersect, permitting higher levels of precision to control transgene expression spatially and temporally.

RECOMBINASE TRANSGENIC MICE: SCREENING FOR MISBEHAVIOR

The most important key for tissue-specific conditional gene modifica-

tion is cell-type specificity of the recombinase transgene. When desired tissue-specific regulatory elements are defined and available, tissue-specific recombinase expression can be achieved by conventional transgenesis by random integration of DNA constructs microinjected into zygotes. In general, however, the expression of transgenes, in terms of specificity and levels of expression, can often be variable among different lines carrying the same transgene, depending on the structure of the transgene integration and chromosomal location. Thus, among multiple transgenic lines that are initially generated, those that show the desired tissue-specificity of recombinase transgene expression need to be carefully prescreened prior to use. When a desired tissue-specific regulatory element is unknown, the recombinase transgene can be introduced into known gene loci that are expressed in the tissue of interest by gene targeting in ES cells (knockin; KI), such that recombinase gene expression is regulated by the endogenous transcriptional control. One can expect in this type of strategy more consistent and predictable expression patterns of the recombinase transgene, and indeed, a number of Cre KI alleles have been confirmed to closely recapitulate endogenous gene expression (Moses et al., 2001; Jamin et al., 2002; Motoike et al., 2003; Heinrich et al., 2004; Tian et al., 2005). The expression levels of a recombinase transgene are likely to depend on the transcriptional activity of the targeted locus. Thus, if a target locus is expressed only at modest levels, then recombinase activity may need to be examined carefully to evaluate whether satisfactory levels of recombination can be achieved.

Although no systematic reports are available for unexpected ectopic expression of recombinase transgenes, such uncontrolled recombinase expression can negatively impact the efficiency of a desired gene modification. This is especially relevant when the gene for tissue-specific modification is essential for the development of a

variety of tissues and animal viability, because widespread recombinase expression may cause gene modifications in multiple tissues and/or embryo death. Unexpected behavior of a recombinase transgene can be observed even in recombinase KI strains in which one would expect more consistent expression. An example of this was reported by Hebert and McConnell (2000), where Cre was targeted into the *Foxg1* (*Bf1*) locus, initially identified as a gene specifically expressed in the developing telencephalon (Tao and Lai, 1992). Examination of recombinase reporter expression revealed that Cre was active in the early developing telencephalon as expected, and in several other tissues where *Foxg1* is known to be expressed. However, they also found unexpected, widespread expression of Cre activity in a substantial number of embryos in Cre reporter assays. They found that genetic background influenced the frequency of this ectopic expression. In addition, ectopic expression was more often observed in *Foxg1Cre*; Z/AP mice than in *Foxg1Cre*; R26R mice, suggesting different susceptibility to recombination, depending upon the target reporter locus. The authors suggested the need for careful examination of in vivo recombinase activity specific to individual conditional mutant loci and genetic backgrounds.

In our own study, we generated multiple lines of transgenic mice expressing Cre under the control of *Six3* sequences in a retina- and ventral forebrain-specific manner (Furuta et al., 2000). We noted that, in addition to variability in tissue-specific Cre activity between transgenic lines, variable leaky Cre expression was detected in individuals within transgenic lines, suggesting that an unknown factor other than transgene integration site was affecting Cre expression. In the course of generating *Bmpr1a* conditional mutant animals, this inconsistent behavior of the *Six3-Cre* transgene significantly reduced the frequency of obtaining the desired tissue-specific conditional mutant animals (Fig. 1A). This is likely that

the widespread expression of the *Six3-Cre* transgene was recombining the *Bmpr1a* floxed allele, causing lethality because *Bmpr1a* $-/-$ mice die around gastrulation (Mishina et al., 1995). Although we did not extensively explore potential genetic background effects, we noticed that the behavior of the *Six3-Cre* transgene was also variable depending upon which mating pairs were employed. We then tested whether overall efficiency of obtaining conditional mutants could be improved by identifying *Six3-Cre* transgenic animals that gave more consistent Cre expression. The typical mating scheme that we used in our study is illustrated in Figure 1. We preferentially used *Six3-Cre* transgenic males heterozygous for a *Bmpr1a* null allele, which were crossed with females homozygous for the floxed *Bmpr1a* conditional allele (Mishina et al., 2002) (Fig. 1A). Before setting up these crosses, we screened *Bmpr1a* null/+; *Six3-Cre*

Although we have no mechanistic explanation for such peculiar behavior of the Cre transgene, preselecting appropriate stud males greatly improved the efficiency of our mating scheme.

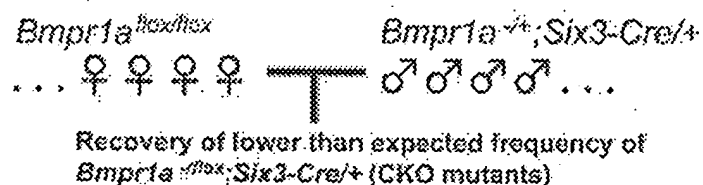
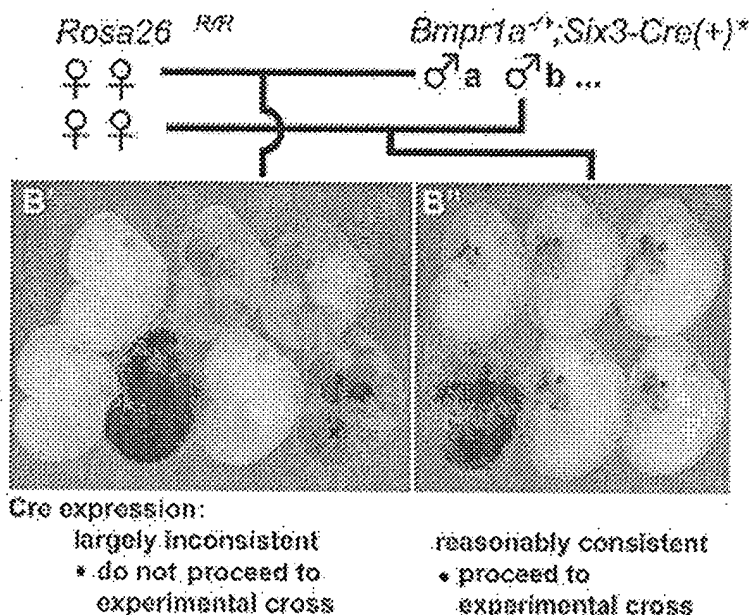
stud male mice for consistent tissue-specific Cre activity in the progeny from crosses with R26R reporter mice (Fig. 1B). The results indicated that some of the *Bmpr1a* null/+; *Six3-Cre* males yielded the desired tissue-specific Cre expression in most of their embryos (Fig. 1B), but other males showed a high frequency of leaky Cre expression (Fig. 1B'). At least two litters of embryos were tested for each *Bmpr1a* null/+; *Six3-Cre* stud male. The

overall efficiency of generating the desired tissue-specific CKO mice followed predictions from the above test crosses with R26R mice. We obtained the desired tissue-specific conditional mutants at a frequency expected from our crosses, whereas for those males that conferred leaky Cre expression, the frequency of the desired tissue-specific CKO animals was greatly reduced. Although we have no mechanistic explanation for such peculiar behavior of the Cre transgene, preselecting appropriate stud males greatly improved the efficiency of our mating scheme. Preselection of mice consistently expressing tissue-specific Cre transgenes is even more critical when the conditional mutant genotype must be combined with (an) other mutant allele(s) (Murali et al., 2005).

TROJAN HORSE: LOXP SITES IN BAC VECTORS

As mentioned above, transgenic mice that express Cre in a tissue-specific manner can be generated using a variety of different methods. One approach to generate tissue-specific Cre transgenic mice is to modify a bacterial artificial chromosome (BAC) clone containing the gene of interest by recombinogenic engineering ("recombineering") in bacteria, introducing Cre into the gene within the clone (Copeland et al., 2001). This method is used when little or nothing is known about the location of tissue-specific regulatory sequences. The assumption is that BACs are large and may contain most, if not all, of the regulatory elements necessary to direct tissue-specific expression. This is not true for all genes, but is a reasonable alternative approach to a Cre KI of the gene of interest by targeting in ES cells. These Cre BAC constructs can then be microinjected into zygotes to generate transgenic mouse lines and characterized using Cre reporter mice.

One concern that has arisen is that many of the mouse BAC libraries have been generated using vectors that contain loxP sites (Fig.

A Experimental cross; no Cre transgene pre-screening**B Test cross; Cre transgene pre-screening**

2A). For example, the mouse BAC libraries RPCI-23 (C57BL/6J) and RPCI-22 (129/SvEv) are derived from pBACe3.6, which contains a wild-type *loxP* and a mutant *loxP511* site (Frengen et al., 2000) (BACPAC Resources Center [BPRC], Children's Hospital Oakland Research Institute [CHORI], Oakland, California; <http://bacpac.chori.org>). These *loxP* sites are features that allow Cre-mediated modifications of the clones. The presence of these BAC vector *loxP* sites can cause potential problems when using Cre BAC transgenes for tissue-specific gene modifications.

Foreign DNA fragments that are microinjected into mouse zygotes typically integrate as head to tail tandem arrays (Palmiter and Brinster, 1986). Therefore, multicopy integration of *loxP*-containing BACs into the mouse genome essentially results in the BACs becoming floxed

at the site of chromosomal integration (Fig. 2B). One study showed that transgenes with *loxP* sites can lead to chromosome damage and loss, creating aneuploidy after exposure to Cre (Lewandoski and Martin, 1997). These authors suggested that if some of the copies of the foreign DNA in the tandem array integrate in opposite orientation, then chromosomal damage can occur. However, if the DNA fragments integrate with all *loxP* sites in the same orientation, then Cre can act to delete all DNA between the *loxP* sites without chromosomal damage.

The presence of *loxP* sites in Cre BAC transgenes can also cause problems, because Cre expression from the BAC will act on the floxed BAC transgene tandem array to delete itself, leading to a subsequent loss of Cre expression (Fig. 2B). In a tissue-specific knockout experi-

Figure 1. Prescreening males for consistent tissue-specific Cre expression. Shown is an example to generate retina-specific *Bmpr1a* conditional mutant mice, using the *Six3-Cre* transgenic mouse line (Furuta et al., 2000). **A:** Males heterozygous for a *Bmpr1a* null allele (Mishina et al., 2002) and the *Six3-Cre* transgene were crossed with *Bmpr1a* *flox/flox* females to obtain conditional mutants (*Bmpr1a* *flox/flox*; *Six3-Cre* *+/+*). During the initial phase of experiments, there was a significantly reduced efficiency for obtaining the desired CKO embryos from this mating scheme. As the *Six3-Cre* transgene was noted to be expressed ectopically in a certain population of embryos (Furuta et al., 2000), death of CKO mutants due to widespread *Bmpr1a* mutation induction was suspected. **B:** Further examination of Cre expression profiles indicated that consistency in tissue-specific expression of the Cre transgene was dependent on the stud males (**B'**, **B''**), prompting the establishment of routine prescreening crosses of stud males with the *Rosa26* reporter (*R26R*) line. Multiple litters of embryos were examined for each stud male. Such a prescreening mating scheme dramatically improved the overall efficiency of obtaining the desired CKO mutants.

ment, one can imagine full recombination or mosaicism of the target floxed allele of interest, depending upon the timing of Cre BAC transgene expression and the subsequent deletion of the BAC tandem array. In addition, depending upon the structure of the integration of the BAC transgene, chromosome damage and aneuploidy is possible (Fig. 2C). Therefore, it is important to determine if the BAC clone you want to recombine with Cre has a *loxP* site in its backbone. If it does, then you will need to disrupt the *loxP* site by an additional round of recombining before generating transgenic mice. Since the number of mouse BAC libraries that are commonly used is still relatively small, it may be possible to develop universal targeting vectors to disrupt *loxP* sites in BAC clones from these libraries.

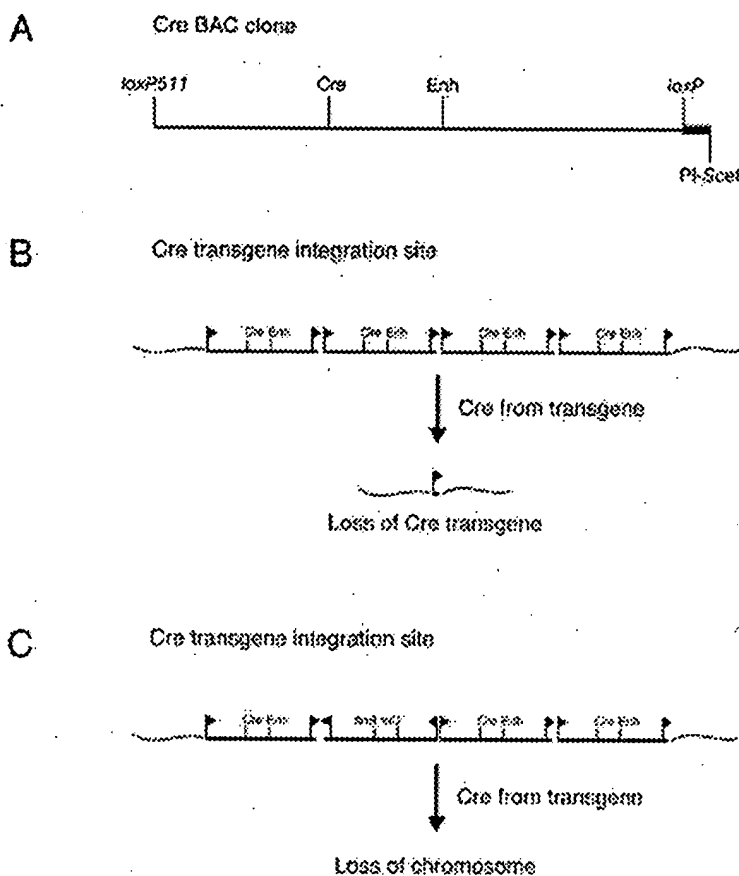


Figure 2. Consequences of *loxP* sites in BAC vectors for Cre BAC transgenes. **A:** Typical BAC clone in which Cre has been introduced into a specific locus by homologous recombination in bacteria. This assumes that there is a tissue-specific transcriptional enhancer (Enh) located on the BAC clone. Many BAC vector backbones (thick line) have *loxP* and *loxP511* sites and rare cutting restriction sites (P1-SceI) to linearize the BAC clones. **B,C:** Idealized multicopy integration of the Cre BAC into a chromosomal locus. **B:** In this example, each BAC is oriented in a head-to-tail tandem array that essentially creates a floxed transgene insert. Cre expression from the transgene would then act upon the tandem array, resulting in excision. **C:** In this example, one of the BACs has integrated in reverse orientation to the other BACs. Cre expression from the transgene would then act upon the BAC array resulting in chromosomal damage and loss. Triangles, *loxP* sites and their orientation.

TIMING CRE ACTIVITY

In conventional recombinase-expressing transgenic mouse lines, temporal regulation of recombinase expression is entirely dependent on the activity of corresponding regulatory elements. The recent development of inducible recombinase transgenic systems has added another level of control of recombinase expression, thereby achieving conditional gene modifications with more precise temporal restrictions. The means to control recombinase activity in vivo under desired temporal regulation is by administration of inducible agents. The first of such systems was developed using a transcriptional regulatory se-

quence of the myxovirus resistance 1 (*Mx1*) gene that was coupled with Cre (Kuhn et al., 1995). The *Mx1* gene, normally involved in defense to viral infection, is silent in healthy mice, but can be induced to high levels in response to pathogenic stimuli such as viral infection. The *Mx1* regulatory element can be artificially induced in a number of tissues, including the liver and spleen, by administration of interferon. While a number of CKO studies have been successfully performed using this inducible Cre system (Hayashi et al., 2004; Le et al., 2004; Schneider et al., 2004; Wells et al., 2004), variable levels of transgene induction by interferon

administration, limited spectrum of responsive cell types, and influence of interferon on endogenous signaling pathways represent potential limitations for the use of this system.

Therefore, it is important to determine if the BAC clone you want to recombineer with Cre has a *loxP* site in its backbone. If it does, then you will need to disrupt the *loxP* site by an additional round of recombineering before generating transgenic mice.

More recently, use of mouse lines expressing ligand-regulated forms of recombinases has allowed a more consistent temporal control of recombinase-mediated gene modification in embryos, as well as in adult tissues. In particular, various mouse lines expressing versions of Cre and Flp recombinases fused to mutant forms of the estrogen receptor (ER) ligand binding domain (LBD) have been generated and used to activate or inactivate gene function in vivo (FlpER [Logie and Stewart, 1995]/CreER [Metzger et al., 1995; Fell et al., 1996; Brocard et al., 1997; Schwenk et al., 1998]). These ER-fused forms of recombinases are insensitive to endogenous β -estradiol, but responsive to the synthetic antagonist 4-hydroxy (4OH)-tamoxifen (TAM), allowing tight control of recombinase activity through ligand-mediated translocation of recombinase proteins from the cytoplasm to the nucleus where they act upon the target DNA sequence. Such ligand-mediated activation of recombinases has been applied in two ways. First, transgenic mouse lines have been

generated that exhibit broad expression of CreER fusions for temporal control of recombinase activity in most tissues (Guo et al., 2002; Hayashi and McMahon, 2002; Seibler et al., 2003). These types of mice have been used to establish optimal doses of TAM administration and to characterize the frequency of induced recombination in a variety of tissues. These broadly expressed recombinase-ER mice will be useful to modify conditional gene loci at different developmental time points, to induce spatially and temporally regulated gene modifications in certain tissues by local administration of inducers, and to modify cells and tissues in vitro (Hayashi and McMahon, 2002).

A second approach is to express recombinase-ER-fusions in tissue-specific manners using tissue-specific regulatory elements (Danielian et al., 1998; Schwenk et al., 1998; Indra et al., 1999; Vasioukhin et al., 1999; Eckardt et al., 2004; Xu et al., 2004). This type of system permits both temporal and spatial control of recombinase activity. Tissue-specific ligand-inducible Cre systems have been successfully used to inactivate the function of a number of genes especially in postnatal tissues, including the skin (McLean et al., 2004), adipocytes (Imai et al., 2004), and heart (van Rijen et al., 2004). In developing embryos, these systems have been particularly useful to define cell lineage relationships (see below).

As inducible recombinase activity in these systems is an important factor to affect the outcome of certain experimental settings, efficiency of ligand-mediated recombination in vivo has to be carefully monitored. Currently, three different derivatives of CreER fusion proteins have been widely used in the mouse: 1) mouse ERTM (G525R) (Danielian et al., 1998; Guo et al., 2002; Hayashi and McMahon, 2002; Zirlinger et al., 2002); 2) human ERT (G521R) (Logie and Stewart, 1995; Metzger et al., 1995; Feil et al., 1996; Brocard et al., 1997; Schwenk et al., 1998; Li et al., 2000; Vooijs et al., 2001); and 3) human ERT2 (G400V/M543A/L544A) (Feil et al., 1996; Indra et al., 1999; Kimmel et al., 2000; Imai

et al., 2001; Seibler et al., 2003). Assays comparing the activity of CreERT with CreERT2 indicate that CreERT2 is ~10-fold more sensitive than CreERT (Indra et al., 1999). A FlpeERT2 system has recently been developed that shows comparable activity to that observed with CreERs in vitro and in vivo (Hunter et al., 2005). In studies to monitor Cre activity using a transgenic line ubiquitously expressing CreERTM, recombination was detectable in many cell types within 24 hr post-TAM injection, peaking by 48 hr in embryos (Hayashi and McMahon, 2002). With a single administration of TAM into pregnant females at 8.5 days postcoitus (8.5 dpc), near complete recombination of the *R26R* reporter locus in many tissues was achieved in the developing embryos at doses of 3–9 mg/40 gm pregnant female body weight. Higher doses within this range, however, were found to affect survival of fetuses at term, presumably due to perturbation of maternal-fetal interactions (Sadek and Bell, 1996). However, administration at later stages (e.g., 13.5 dpc or thereafter) did not appear to affect fetal survival. In the adults, similar doses did not overtly affect animal physiology, but near-complete recombination of the *R26R* allele was achieved in many tissues after administration of the inducer for five consecutive days. In this study, these levels of efficient recombination were achieved using TAM, which is metabolized by the liver to the active inducer 4-OH-TAM. TAM is more soluble in solution, and is much less costly than 4-OH-TAM. Recent preliminary studies have indicated that apparently comparable levels of Cre-mediated recombination can be induced by 4-OH-TAM and TAM in mouse embryos (Dr. Randy Johnson, personal communication).

Other means for temporal control of Cre activity have also been developed. The fusion of a mutant progesterone receptor (PR) LBD linked to the C-terminus of Cre, termed CrePR, has been found to be largely insensitive to endogenous progesterone, but responsive

to the synthetic steroid RU486 (Kellendonk et al., 1996). The prototype CrePR1 has been shown effective in vivo, in adult epidermis to model human skin disorders (Arin et al., 2001; Cao et al., 2001), and has also been suggested to be effective in utero (Zhou et al., 2002). CrePR1 has, however, been found to be somewhat leaky, with detectable recombinase activity in the absence of inducer. An improved version of CrePR, termed Cre*PR, has been shown to have lower background activity and better sensitivity to RU486 (Wunderlich et al., 2001). The development of ER and PR versions of recombinases provides the opportunity for the temporal induction of multiple genetic modifications; e.g., use of Cre-PR and FlpER controlled by RU486 and TAM, respectively, will allow simultaneous or sequential gene activation/inactivation in the same animal.

RECOMBINASE-BASED FATE MAPPING IN MICE

Knowledge of the relationships between progenitor or stem cells and their cellular progeny provides a foundation for understanding how tissues and organs are formed. These cell lineage relationships can be determined by fate mapping. Classically, embryonic cells were labeled with vital dyes and the marked cells followed through development to create fate maps. This approach has limited use in the mouse because development of the embryo occurs within the uterus, hindering access to the embryo for experimental manipulation. DNA recombinases, notably Cre, are currently being used in the mouse to map the fates of embryonic cells. These fate-mapping studies have been enabled by the development of the various Cre reporter mouse strains mentioned above. Basically, mice that bear Cre transgenes or knockins are examined for Cre expression by crosses with Cre reporter mice. Cre expression is measured by reporter expression at some later stage of development (Fig. 3A and B).

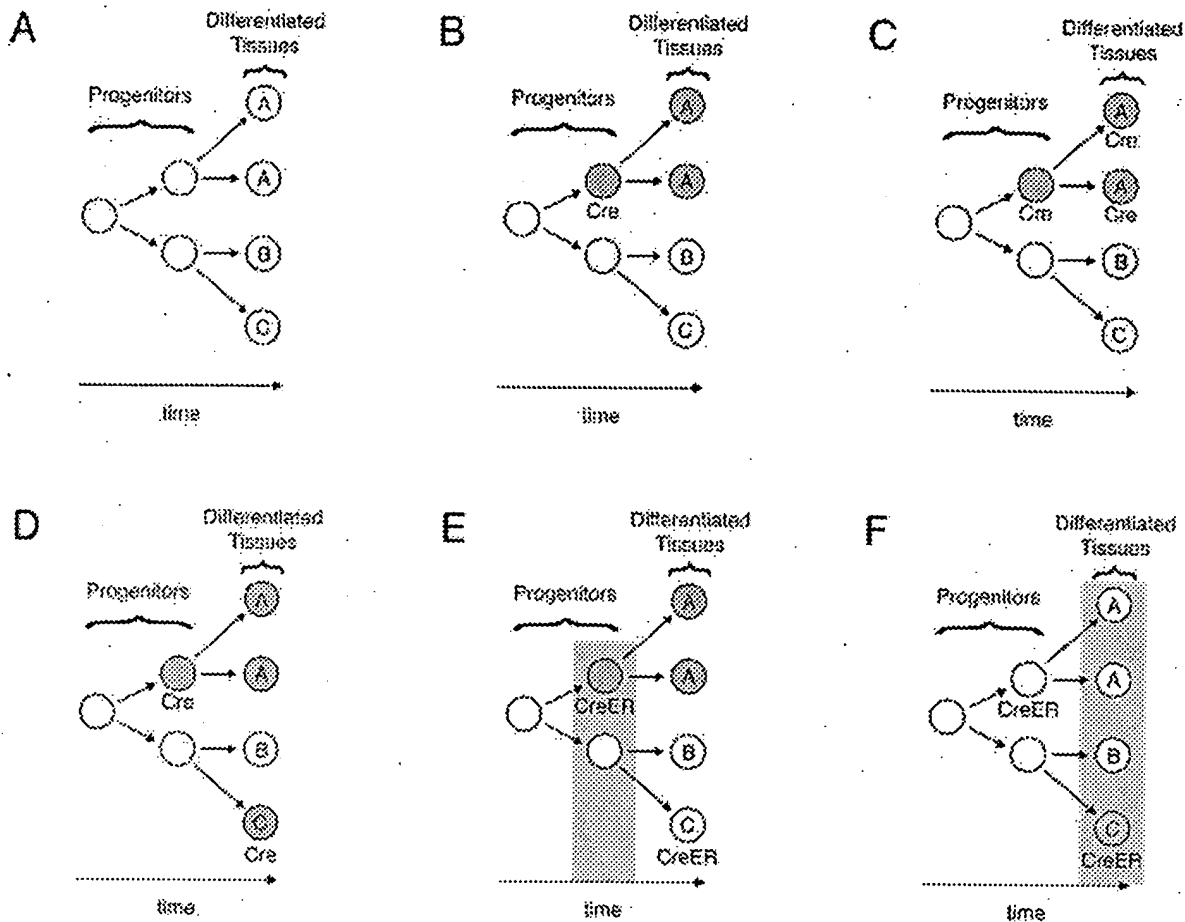


Figure 3. Recombinase-based fate mapping in the mouse. **A:** Diagram showing progenitor cells and their lineage relationships with the cellular progeny to form differentiated tissues A–C. **B:** In this situation, Cre expression from a transgene occurs only in the progenitor cells that give rise to the cells of tissue A. Thus, incorporation of a Cre reporter would result in reporter expression in the shaded cells, i.e., A progenitors and A cell types. This could be used to create a fate map. **C:** In this situation, Cre expression from a transgene occurs in progenitor cells that give rise to tissue A. In addition, the Cre transgene is active in A cell types. Thus, incorporation of a Cre reporter would result in reporter expression in A progenitors and A cell types. A fate map cannot be determined. **D:** In this situation, Cre expression from a transgene occurs in progenitor cells that give rise to tissue A. In addition, the Cre transgene is active in C cell types. Thus, incorporation of a Cre reporter would result in reporter expression in A progenitors, A and C cell types, a collation of fate maps. **E:** In this situation, CreER substitutes for Cre in (D). CreER transgene expression occurs in progenitor cells that give rise to tissue A and also C cell types. Administration of TAM (shaded box) only activates CreER in A progenitors. Incorporation of a Cre reporter would result in reporter expression only in A cell types. **F:** This is the same situation as in (E) but TAM (shaded box) is administered later, activating CreER only in C cell types. Thus, a Cre reporter would only mark C cell types.

These recombinase-based fate-mapping strategies have become very popular (Zinyk et al., 1998; Chai, 2000; Epstein et al., 2000; Zirlinger et al., 2002; Awatramani et al., 2003; Motoike et al., 2003; Zhang et al., 2003). However, their utility depends upon a set of fundamental principles. First, the entire pattern of Cre expression from the transgene or KI must be known in the relevant tissues throughout development at the level of Cre RNA or protein. This requires either Cre in situ hybridization or immunohistochemistry to define the temporal

and spatial pattern of expression. This has been simplified somewhat by the creation of a GFP-Cre fusion protein, allowing one to follow Cre protein by GFP fluorescence (Heinrich et al., 2004). Second, the temporal and spatial pattern of Cre reporter expression (e.g., *lacZ* in the case of R26R) must be determined. Comparisons of Cre RNA or protein expression with Cre reporter expression then allows one to make conclusions about cell lineage relationships. Another aspect of this system is that examination of Cre reporter expression at one time

point is actually a collation of cellular progeny generated from all of the progenitor cells that have expressed Cre and activated the reporter. These progenitor cells may be within the same lineage but at different developmental stages or different cell lineages (Fig. 3C). Thus, examining only one terminal stage of development may provide a misleading fate map.

To get around the shortcoming of using a constitutively active recombinase, fate mapping strategies have recently been modified to incorporate drug-inducible recombi-

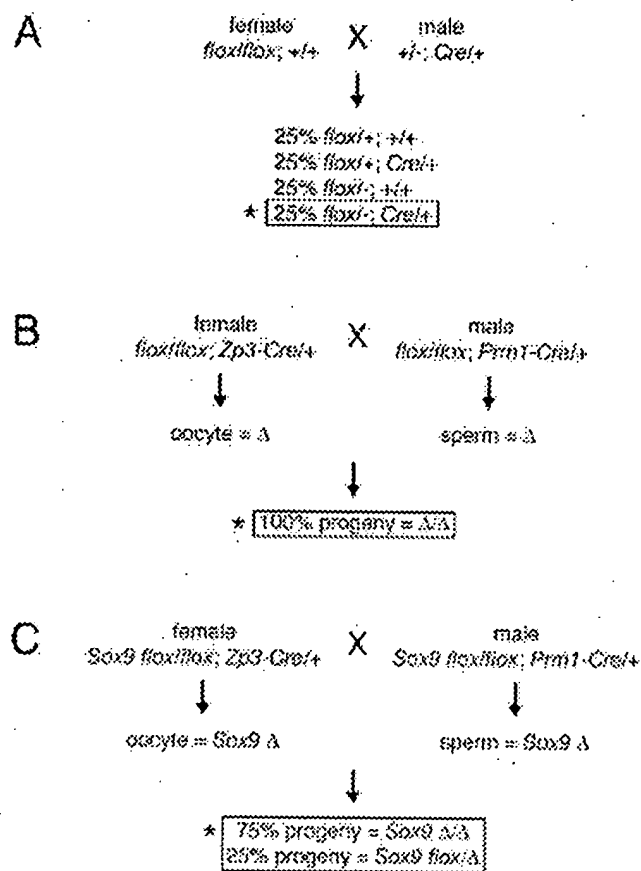


Figure 4. Manipulating Mendelian ratios using germ cell-specific Cre transgenes. **A:** Typical cross to yield 25% CKO mutants, using a Cre transgene and null and flox alleles. **B:** Conceptual cross, using germ cell-specific Cre transgenes with a floxed allele to generate only recombined (Δ) allele-carrying gametes. In theory, only Δ/Δ mice are generated from this cross. **C:** Cross used to generate *Sox9* Δ/Δ mice (Chaboissier et al., 2004). The particular *Prm1*-Cre transgenic mouse line is not 100% efficient for recombining the *Sox9* flox allele. Thus, most of the progeny are *Sox9* Δ/Δ , but *Sox9* $\Delta/+$ controls are also generated.

nases (Danielian et al., 1998; Guo et al., 2003; Sgaier et al., 2005). The most popular drug-inducible recombinases are CreER and CrePR, that are induced by TAM and RU486, respectively (see above) (Nagy, 2000). In addition, a tamoxifen-inducible Flp recombinase has recently been reported (Hunter et al., 2005). In this type of strategy, the time and extent of exposure to the inducing drug results in activation of the recombinase only in those cells expressing Cre protein during the time of exposure (Fig. 3D and E). By varying the timing of the drug induction, one can create temporal fate maps, providing more precise information about the relationships of progenitors and their cellular progeny.

MANIPULATING MENDELIAN RATIOS USING GERM CELL-SPECIFIC RECOMBINATION OF CONDITIONAL ALLELES

One of the difficulties for studying a recessive embryonic lethal phenotype in mice is that only one-quarter of the progeny from heterozygous intercrosses will be homozygous mutant. The same 25% frequency is obtained from crosses when performing a routine tissue-specific knockout (Fig. 4A). In addition to this relatively low frequency, each embryo usually needs to be genotyped for analysis, hindering studies requiring large numbers of mutant embryos (e.g., microarray

or biochemical studies). Of course, one could just use brute force and generate a lot of crosses and genotype embryos prior to combining them for analysis. However, a genetic system has been devised to alter the frequencies of genotypes for progeny generated from matings of mice with conditional alleles (Fig. 4B). This system creates the illusion that Mendel's laws are being broken, but in reality they are still intact. One use of this system is to increase the frequency of a particular genotype obtained from crosses. This system has also been exploited to bypass an unusual heterozygous lethality (Bi et al., 2001; Akiyama et al., 2002; Chaboissier et al., 2004).

The system utilizes transgenic mouse lines that express Cre recombinase in germ cells, i.e., oocytes and spermatogenic cells. Zona pellucida glycoprotein 3 (*Zp3*) is expressed in growing oocytes (Epifano et al., 1995). In one study, a 6.5-kb fragment of the *Zp3* promoter region directed Cre recombinase to oocytes of female transgenic mice (Lewandoski et al., 1997). In a subsequent study, a 6.0-kb fragment from the *Zp3* promoter region was also able to direct Cre recombinase expression only in oocytes of female transgenic mice (de Vries et al., 2000). Protamine 1 (*Prm1*) is expressed in haploid round spermatids (Hecht et al., 1986). A 652-bp fragment from the *Prm1* promoter region directed Cre recombinase expression only in spermatids of male transgenic mice (Peschon et al., 1987; O'Gorman et al., 1997). These *Prm1*-Cre transgenic males were otherwise normal and fertile. However, in another *Prm1*-Cre transgenic mouse strain that used a 4.2-kb promoter region, males derived from female founders were sterile (Schmidt et al., 2000). Apparently, Cre-mediated chromosomal rearrangements utilizing "pseudo-*loxP*" sites caused embryo loss. It was suggested that these Cre-mediated rearrangements were caused by higher levels or timing of Cre expression. Together, these oocyte- and spermatid-specific Cre recombinase lines are combined with *flox* alleles in

various breeding schemes, depending upon the particular situation, to produce oocytes and sperm carrying recombined (i.e., knock-out) alleles of the gene of interest (Fig. 4B).

This system was used recently to bypass a neonatal haplolethality caused by a null mutation in *Sox9* (Akiyama et al., 2002; Chaboissier et al., 2004). Humans that are heterozygous for *SOX9* mutations develop a severe chondrodysplasia called campotomic dysplasia (Foster et al., 1994; Wagner et al., 1994). Mice heterozygous for a targeted null allele of *Sox9* also develop a similar skeletal syndrome that causes lethality soon after birth (Bi et al., 2001; Akiyama et al., 2002; Kist et al., 2002). Another haplolethality has been observed when mice are heterozygous for a targeted *Vegfa* mutation (Carmeliet et al., 1996; Ferrara et al., 1996). *Vegfa* heterozygous mutant mice die between 9.5 and 10.5 dpc because of abnormal blood vessel development. As one can imagine, these heterozygous lethal phenotypes make it very difficult to generate homozygous mutants.

To generate *Sox9* homozygous mutants, the germ cell-specific recombination system of conditional alleles mentioned above was used (Akiyama et al., 2002; Chaboissier et al., 2004). *Sox9 flox/flox*; *Zpe-Cre* tg/+ females were created as were *Sox9 flox/flox*; *Prm1-Cre* tg/+ males that were viable, normal, and fertile (Fig. 4C). As germ cells formed in these mice, Cre recombinase was expressed and acted on the *flox* allele to convert it to the recombined allele. The result was the generation of *Sox9* homozygous mutants. These mutants died at 11.5 dpc with neural tube and cardiovascular defects. Interestingly, the system was not completely efficient; only about 75% of the resulting embryos were *Sox9* Δ/Δ , the others were *Sox9 flox/\Delta*. This is because the particular *Prm1-Cre* line is not completely efficient in converting the *Sox9 flox* allele to -.

These 11.5 dpc *Sox9* Δ/Δ embryos provided an opportunity to study the role of *Sox9* in testis dif-

ferentiation that occurs in the mouse at 12.5 dpc (Chaboissier et al., 2004). If wild-type 11.5 dpc XY urogenital ridges are explanted in vitro for organ culture for two days, they will differentiate into testes. Therefore, the urogenital ridges from the above crosses were explanted in vitro for organ culture for two days. The XY *Sox9* Δ/Δ gonads did not form testes, but rather they expressed markers of ovarian differentiation, suggesting that *Sox9* is essential for testis formation. These studies highlight the unique utility of the germ cell-specific recombination of conditional alleles system. The use of this system to generate mutant embryos at high frequency for molecular or biochemical studies has yet to be reported.

CONDITIONAL COMPOUND MUTANTS: TISSUE-SPECIFIC GENETIC REDUNDANCY AND BEYOND

While CKO of a number of genes has led to informative phenotypes, recent studies have also begun to uncover remarkable degrees of redundancies between related genes in some tissue-specific contexts. Recently, an increasing number of studies have combined multiple mutant genetic backgrounds involving CKO mutant alleles, which have proved to be very powerful, not only to elucidate genetic redundancy, but also to uncover novel insights into the roles of various genetic pathways. One of the first series of studies involving CKO of multiple gene loci are those demonstrating requirements for fibroblast growth factor (FGF) signaling in limb development. Members of the FGF family of secreted proteins have been implicated as signals required for limb development derived from the apical ectodermal ridge (AER), an important signaling center for limb outgrowth and patterning (Niswander et al., 1993; Fallon, 1994). In particular, both *Fgf4* and *Fgf8* were proposed to mediate critical functions of the AER based on their specific expres-

sion in this tissue. Genetic analysis of *Fgf4* and *Fgf8* function in the limb, however, required a tissue-specific inactivation approach because nullizygosity for either gene alone caused early embryonic lethality (Feldman et al., 1995; Sun et al., 1999). Conditional loss of *Fgf4* function in the AER had no effect on limb development (Moon et al., 2000; Sun et al., 2000), whereas inactivation of *Fgf8* resulted in hypoplasia of all three limb segments (Lewandoski et al., 2000; Moon and Capecchi, 2000). Inactivating both *Fgf4* and *Fgf8* in the AER produced more severe phenotypes than inactivating *Fgf8* alone in the AER, genetically demonstrating redundant roles for these *Fgf* genes (Sun et al., 2002; Boulet et al., 2004). While these studies confirmed an absolute requirement for *Fgf4* and *Fgf8* for limb outgrowth, the phenotypes of the *Fgf4*; *Fgf8* AER-conditional, conditional double KO (AER-CCDKO) were not equivalent to that induced by AER removal. Surgical removal of the AER results in extensive cell death of distal limb mesenchyme (Sun et al., 2002), whereas simultaneous loss of *Fgf4* and *Fgf8* in the AER appeared to affect cell survival of the more proximal mesenchyme. The observed phenotypes of the *Fgf4*; *Fgf8* AER-CCDKO mutants appear to be compatible with the notion that a principal function of FGF signaling from the AER is to ensure that enough progenitor cells are available to form each element of the limb skeleton, by promoting cell survival proximally. These genetic studies substantiated a novel model for AER function in limb outgrowth, leading to a reassessment of the classical progress zone model that had served as a paradigm of the field for over 30 years (Wolpert, 2002). It should be noted that AER expression of two additional FGF members, *Fgf9* and *Fgf17* (Xu et al., 2000; Colvin et al., 2001) appeared relatively normal in the absence of *Fgf4* and *Fgf8* (Sun et al., 2002). This indicates that *Fgf9* and/or *Fgf17* expression is independent of *Fgf4* and *Fgf8* during limb development. Elucidation of the potential

roles of these other AER FGs awaits future genetic studies.

Bone morphogenetic proteins (BMPs) of the TGF- β superfamily are another class of secreted signaling molecules known to play pivotal roles during embryogenesis (Zhao, 2003). These extracellular ligands signal through their specific cell surface transmembrane serine-threonine kinase receptor complexes comprised of type-I and type-II receptors. The type-I receptors primarily dictate the biological output, as they are responsible for activating intracellular signaling mediated by the Smad family of proteins. Among seven TGF- β type-I receptors known in higher vertebrates, three receptors, *Acvr1* (also known as *Alk2*), *Bmpr1a* (*Alk3*), and *Bmpr1b* (*Alk6*), can activate Smads dedicated for BMP-mediated signaling, as opposed to canonical TGF- β and activin signaling (Mishina, 2003). While these BMP receptors are thought to share common intracellular signaling components, it is not entirely clear how the relatively limited number of BMP receptors control a diverse array of biological effects in vivo. Several recent studies suggested that *Bmpr1a* and *Bmpr1b* play distinct roles in vivo. For example, expression of constitutively active forms of these receptors both in transgenic mice and in neural stem cells suggested that *Bmpr1a* regulates proliferation in the developing central nervous system (CNS), whereas *Bmpr1b* controls apoptosis and terminal differentiation (Panchision et al., 2001).

Conventional KO studies in the mouse, on the other hand, have provided only limited genetic evidence to establish the role of BMPs in neural tube development, due to early lethality in some of BMP signaling mutants and potential redundancy between related signaling components. For example, homozygous null mutants for either *Acvr1* or *Bmpr1a* are lethal around gastrulation (Mishina et al., 1995, 1999; Gu et al., 1999), whereas *Bmpr1b* homozygous mutants are viable without overt abnormalities in the CNS (Baur et al., 2000; Yi et al., 2000). Recently, by combining

a neural tube-specific *Bmpr1a* CKO and *Bmpr1b* null mutant genetic backgrounds (conditional, null double knockout, CNDKO), studies have demonstrated that these BMP receptors act largely redundantly in CNS development (Wine-Lee et al., 2004). BMPs have been proposed to mediate signals for the establishment of regional identities of neural progenitors in the dorsal spinal cord (Liem et al., 1997). While elimination of *Bmpr1a* function alone or *Bmpr1b* nullizygosity did not lead to noticeable changes in dorsal spinal cord patterning, combining these mutant genetic backgrounds to eliminate the function of both of these receptors resulted in loss of the two dorsal-most populations of sensory interneurons, DI1 and DI2. While the genesis of more ventrally-derived DI3 and DI4 was unaffected, an expansion of these cell types at the expense of the loss of DI1 and DI2 was observed in the *Bmpr1a/Bmpr1b* neural CNDKO mutants. Other dorsal and ventral populations were unchanged, identifying the cell types that absolutely require BMP signaling for specification mediated by these two receptors (Wine-Lee et al., 2004).

If related growth factor receptor family members, such as *Bmpr1a* and *Bmpr1b*, indeed function redundantly, how can the qualitatively limited nature of the signaling pathways mediated by redundant receptors confer certain levels of complexity to govern multiple biological effects in vivo? Our recent studies, using a *Bmpr1a/Bmpr1b* retina CNDKO similar to that described above, have revealed that multiple developmental programs that simultaneously operate in individual cells are regulated at different threshold levels of BMP signaling that can be genetically titrated by the total level of overall functional BMP receptors (Murali et al., 2005). Using the *Six3-Cre* transgenic mice described above, we created CKO mutant animals that lack *Bmpr1a* in the developing retina, but no apparent eye abnormality was detected in these animals. Further reduction of BMP receptor activity by removing one functional copy of *Bmpr1b* in the *Bmpr1a* ret-

ina-CKO mutant background again resulted in no detectable changes in eye morphology (Fig. 5A) or generation of various retinal cell types. However, these animals were found to exhibit abnormal retinal dorsal-ventral (D-V) patterning (Fig. 5B-D). This suggested that one copy of *Bmpr1b* is sufficient to maintain eye morphogenesis and differentiation, but not to specify spatial patterning of the developing retina. In the *Bmpr1a/Bmpr1b* retina CNDKO mutants, lacking both of these genes resulted in severe eye defects (Fig. 5E), characterized by reduced growth of embryonic retina and failure of retinal neurogenesis. These findings are consistent with the notion that *Bmpr1a* and *Bmpr1b* play redundant roles during retinal development. In addition, a spectrum of phenotypes correlated with the levels of loss of BMP receptor function suggests different threshold levels of BMP signaling regulate distinct developmental programs such as patterning, growth, and differentiation of the retina (Murali et al., 2005). This provides one model as to how BMP signaling can regulate diverse cellular responses during organogenesis in vertebrates. The mechanisms by which individual cells sense the overall receptor-mediated signaling and translate it to elicit qualitatively distinct genetic programs may have implications for other growth factor signaling systems.

Genetic redundancy has also been a challenge for investigators to generate mouse models that precisely mimic certain human diseases. One prominent example is a series of attempts to model human retinoblastomas in the mouse. Retinoblastomas develop early in life and at high frequency in individuals heterozygous for a germline mutation of the retinoblastoma (*RB*) gene, the first known tumor suppressor gene identified (Friend et al., 1986; Fung et al., 1987; Lee et al., 1987). Sporadic retinoblastomas also invariably have somatic mutation in the *RB* gene. Although humans who have inherited germline *RB* heterozygous mutations are at some increased risk for tumors

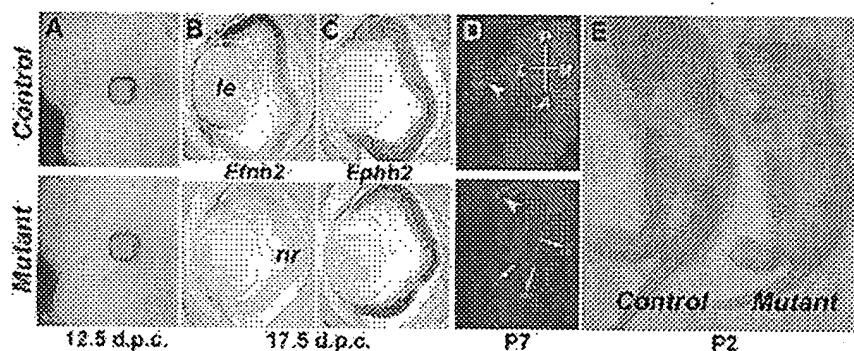


Figure 5. Tissue-specific gene redundancy revealed by retina-specific *Bmpr1a* CNDKO **A–D:** Retinal D–V patterning defects in *Bmpr1a*^{lox/+}; *Bmpr1b*^{+/+}; *Six3-Cre*/+ mutant mice (Mutant). Control littermates shown are representatives of the *Bmpr1a*^{lox/+}; *Bmpr1b*^{+/+}; *Six3-Cre*/+ or *Bmpr1a*^{lox/+}; *Bmpr1b*^{+/+}; *Six3-Cre*/+ genotypes. **A:** The *Bmpr1a*^{lox/+}; *Bmpr1b*^{+/+}; *Six3-Cre* mutants show no overt morphological defects in the eye (bottom). **B,C:** Coronal sections of embryonic eyes with dorsal on top. In the mutants, the expression of a dorsal marker *Efrnb2* is lost (**B**; bottom), while transcripts of *Ephb2*, normally ventrally enriched, are now expanded throughout the retina (**C**; bottom). **D:** Analysis of retinotectal axon projections in the superior colliculus of the midbrain in postnatal day 7 (P7) animals. Focal injection of Dil into the dorsal retina of the left eye reveals a single termination zone of dorsal retinal ganglion cell axons in a lateral-posterior region of the contralateral (right) superior colliculus in control animals (top; arrowhead). In contrast, in the mutants, several ectopic termination zones are seen (bottom; arrows), in addition to a normal lateral-posterior spot (bottom; arrowhead), characteristic defects associated with D–V patterning abnormality. The axes in the right superior colliculus are indicated: A, anterior; L, lateral; M, medial; P, posterior. **E:** The *Bmpr1a*^{lox/+}; *Bmpr1b*^{+/+}; *Six3-Cre*/+ CNDKO mutants exhibit an eyeless phenotype at birth due to severe degeneration during embryogenesis. Abbreviations: le, lens; nr, neuroretina.

other than retinoblastoma, such as osteosarcomas (Eng et al., 1993), the retina appears especially sensitive to *RB* loss. The reason for this tissue sensitivity is unknown. In addition, the cell of origin of retinoblastomas has been controversial. Given apparent developmental origins of retinoblastoma (Salim et al., 1998), elucidating the role of *RB* in normal retinal development should help to explain the mechanisms and origins of this tumor. However, early attempts to genetically model retinoblastomas in the mouse revealed intrinsic difficulty. First, unlike human, germ-line *Rb*^{+/-} mice never develop retinoblastoma, although they tend to develop other types of tumors, including pituitary and thyroid tumors at high frequency (Jacks et al., 1992). Second, *Rb* homozygous mutant embryos die after midgestation with defects in erythropoiesis and placental development (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1994; Wu et al., 2003). Although generation of mouse chimeras composed of wild-type and *Rb* homozygous mutant cells allowed survival to adulthood, these mice

also do not develop retinoblastoma (Maandag et al., 1994; Williams et al., 1994). Recently, several groups generated *Rb* retina CKO mice (Chen et al., 2004; MacPherson et al., 2004; Vooijs et al., 2002; Zhang et al., 2004). Although some differences in phenotype apparently depend on the timing and cell type of *Rb* deletion, the studies collectively revealed that *Rb* function is essential for the specified retinal precursors to exit the cell cycle correctly and survival of certain retinal cell types, including retinal ganglion cells, photoreceptors and bipolar cells. However, again, these *Rb* retina CKO mutants did not develop retinoblastoma, even on a *Trp53* mutant (CNDKO) background (MacPherson et al., 2004). These results were consistent with the hypothesis that other related *Rb* gene family members, *p107* (*Rbl1*) and *p130* (*Rbl2*), may compensate for the loss of *Rb* in the mouse. In contrast to the essential role of *Rb* during development, *p130*^{-/-} and *p107*^{-/-} mice survive to adulthood without mutant phenotypes (Cobrinik et al., 1996; Lee et al., 1996). Null mutations for

these *Rb*-related genes were then combined with the *Rb* retina CKO mutants. The resulting CNDKO animals indeed showed retinal dysplasia or retinoblastoma (Chen et al., 2004; MacPherson et al., 2004). In both *Rb/p107* and *Rb/p130* retina CNDKO mutants, retinal progenitor cell proliferation and precursor specification did not appear affected. Most significant was an aberrant cell proliferation of all specified retinal precursor cell types examined even postnatally, consistent with perturbation of cell cycle exit. Subsequently, there was the elimination of some ectopically proliferating precursor cell populations, including those fated to become retinal ganglion cells, photoreceptors, and bipolar cells. In contrast, other cell types, including amacrine and horizontal neurons, and Müller glial cells survive. In the retinas of *Rb/p107* retina CNDKO mice, these cells appear to exit the cell cycle eventually by postnatal day 30 (P30) and terminally differentiate (Chen et al., 2004). The retinoblastomas that arise in both *Rb/p107* and *Rb/p130* retina CNDKO mutants possess inner nuclear layer (INL) characteristics predominantly of the amacrine lineage. These studies have provided a novel model for the development of retinoblastoma, implying that tumors arise from a subset of the INL precursor cell population that are death-resistant under perturbation of *Rb*-dependent cell cycle exit, rather than undifferentiated progenitor cells. These inheritable models of retinoblastomas will provide versatile resources for the analysis of the cell-of-origin of this tumor, for the elucidation of the genetic events during tumor progression, and for comparison of the molecular features in mouse versus human retinoblastomas.

Combining conditional mutants with other mutant backgrounds is not only useful to elucidate genetic redundancy, but also to provide a versatile system to explore interactions of distinct genetic pathways in specific biological contexts. It has often been difficult to genetically determine whether multiple genetically interacting signaling path-

ways act consecutively or function in parallel to impinge upon certain developmental processes by combining conventional KO mutants. Some recent studies using combinations of compound CKO mutants have started to explore epistatic relationships of multiple genetic pathways in tissue-specific contexts. One such study focused on embryonic limb development, where multiple signaling centers are involved in the regulation of axis patterning. Those include the AER that controls proximal-distal elongation, the zone of polarizing activity (ZPA) responsible for anterior-posterior patterning, and the limb bud ectoderm to direct D-V axis formation. In addition to FGF signaling discussed above, other peptide growth factor signaling pathways are implicated in the formation and activities of these limb patterning signaling centers.

BMP signaling has been implicated in the negative regulation of the AER and D-V patterning of the limb bud (Ng et al., 1999). For example, expression of a constitutively active form of *Bmpr1a* caused ectopic expression of *Fgf8*, an AER signaling component, and the ventral patterning regulator *En1* (Pizette et al., 2001). Consistent with this observation, conditional inactivation of *Bmpr1a* in limb ectoderm, including the AER, impairs both AER formation and D-V patterning of the limb, and perturbs the expression of *Fgf8* and *En1* (Ahn et al., 2001). Wnt signaling is another component that participates in various aspects of limb patterning (Capdevila and Izpisua Belmonte, 2001). More recently, the function of β -catenin, a key mediator of Wnt signaling, was disrupted specifically in early hindlimb bud ectoderm (Soshnikova et al., 2003). The resulting β -catenin CKO mutants, depending on the timing and extent of Cre-mediated recombination, exhibited a variety of phenotypes ranging from complete loss of the hindlimbs to truncation of distal skeletal elements and/or absence of digits. These defects correlated with attenuation of the expression of *Fgf8* and *Bmp4*, indicating disruption of AER formation. Notably, mildly af-

fected hindlimbs showed loss of ventral and duplication of dorsal structures, as indicated by the absence of ventral dermis and presence of circumferential nails. This was also associated with loss of *En1* expression and ectopic *Wnt7a* expression in the ventral ectoderm. Also in this study, a conditional gain-of-function allele of β -catenin (Harada et al., 1999) activated by the same Cre transgene was used, such that the effect of elevated β -catenin signaling was examined. Conditional activation of β -catenin caused ventral expansion of *Fgf8* and *Bmp4*, associated with overall expansion of limb bud outgrowth, opposite to loss-of-function effects (Soshnikova et al., 2003). Striking similarity in the defects observed both in *Bmpr1a* and β -catenin CKO mutants, i.e., defective AER formation and dorsalization of the ventral limb, prompted the hypothesis that BMP and Wnt signaling pathways cooperate to regulate these patterning processes. In order to investigate a potential epistatic relationship between BMP and Wnt signaling, *Bmpr1a* CKO and gain-of-function β -catenin backgrounds were then combined (Soshnikova et al., 2003). Remarkably, activated β -catenin rescued induction of AER markers and allowed expansion of the limb bud on the *Bmpr1a* CKO background, suggesting that Wnt signaling mediated by β -catenin acts downstream of *Bmpr1a*-mediated signaling in AER formation. In contrast, activated β -catenin expression did not rescue ectopic dorsal characters by the loss of *Bmpr1a*. Thus, β -catenin signaling may act upstream or in parallel to BMP signaling in limb D-V patterning. The use of such combinations of tissue-specific inactivating and activating mutant alleles will become useful approaches to elucidate complex genetic network underlying certain developmental events.

CONCLUDING REMARKS

Technical aspects of tissue-specific gene modification approaches are still improving, and these approaches will remain a versatile

means to elucidate gene function and address important biological questions. In addition, since the completion of the mouse genome sequencing project, a portion of the mouse genetics field has been moving toward saturating the genome with random and targeted mutations using conventional approaches (Justice et al., 1999; Hansen et al., 2003; Stryke et al., 2003; Austin et al., 2004). In the long term, future analyses of the mutant resources from these large-scale efforts will be integrated with those from the tissue-specific gene modification techniques discussed above, which will further increase the overall synthesis of the entire system of mouse genetics beyond our current imagination.

REFERENCES

- Ahn K, Mishina Y, Hanks MC, et al. 2001. BMPR-1A signaling is required for the formation of the apical ectodermal ridge and dorsal-ventral patterning of the limb. *Development* 128:4449-4461.
- Akiyama H, Chaboissier M-C, Martin JF, et al. 2002. The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. *Genes Dev* 16:2813-2828.
- Arin MJ, Longley MA, Wang XJ, et al. 2001. Focal activation of a mutant allele defines the role of stem cells in mosaic skin disorders. *J Cell Biol* 152:645-649.
- Austin CP, Battey JF, Bradley A, et al. 2004. The knockout mouse project. *Nat Genet* 36:921-924.
- Awatramani R, Soriano P, Mai JJ, et al. 2001. An F1p indicator mouse expressing alkaline phosphatase from the ROSA26 locus. *Nat Genet* 29:257-259.
- Awatramani R, Soriano P, Rodriguez C, et al. 2003. Cryptic boundaries in roof plate and choroid plexus identified by intersectional gene activation. *Nat Genet* 35:70-75.
- Baur ST, Mai JJ, Dymecki SM. 2000. Combinatorial signaling through BMP receptor IB and GDF5: shaping of the distal mouse limb and the genetics of distal limb diversity. *Development* 127:605-619.
- Bi W, Huang W, Whitworth DJ, et al. 2001. Haploinsufficiency of Sox9 results in defective cartilage primordia and premature skeletal mineralization. *Proc Natl Acad Sci USA* 98:6698-6703.
- Boulet AM, Moon AM, Arenkiel BR, et al. 2004. The roles of Fgf4 and Fgf8 in limb bud initiation and outgrowth. *Dev Biol* 273:361-372.

- Branda CS, Dymecki SM. 2004. Talking about a revolution: The impact of site-specific recombinases on genetic analyses in mice. *Dev Cell* 6:7-28.
- Brocard J, Warot X, Wendling O, et al. 1997. Spatio-temporally controlled site-specific somatic mutagenesis in the mouse. *Proc Natl Acad Sci USA* 94:14559-14563.
- Cao T, Longley MA, Wang XJ, et al. 2001. An inducible mouse model for epidermolysis bullosa simplex: implications for gene therapy. *J Cell Biol* 152:651-656.
- Capdevila J, Izpisua Belmonte JC. 2001. Patterning mechanisms controlling vertebrate limb development. *Annu Rev Cell Dev Biol* 17:87-132.
- Carmeliet P, Ferreira V, Breier G, et al. 1996. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 380:435-439.
- Chaboissier M-C, Kobayashi A, Vidal VIP, et al. 2004. Functional analysis of Sox8 and Sox9 during sex determination in the mouse. *Development* 131:1891-1901.
- Chai Y. 2000. Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. *Development* 127:1671-1679.
- Chen D, Livne-bar I, Vanderluit JL, et al. 2004. Cell-specific effects of RB or RB/p107 loss on retinal development implicate an intrinsically death-resistant cell-of-origin in retinoblastoma. *Cancer Cell* 5:539-551.
- Clarke AR, Maandag ER, van Roon M, et al. 1992. Requirement for a functional Rb-1 gene in murine development. *Nature* 359:328-330.
- Cobrinik D, Lee M, Hannon G, et al. 1996. Shared role of the pRB-related p130 and p107 proteins in limb development. *Genes Dev* 10:1633-1644.
- Colvin JS, Green RP, Schmahl J, et al. 2001. Male-to-female sex reversal in mice lacking fibroblast growth factor 9. *Cell* 104:875-889.
- Copeland NG, Jenkins NA, Court DL. 2001. Recombineering: a powerful new tool for mouse functional genomics. *Nat Rev Genet* 2:769-779.
- Danielian PS, Muccino D, Rowitch DH, et al. 1998. Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. *Curr Biol* 8:1323-1326.
- de Vries WN, Binns LT, Fancher KS, et al. 2000. Expression of Cre recombinase in mouse oocytes: a means to study maternal effect genes. *Genesis* 26:110-112.
- Ding H, Wu X, Nagy A. 2002. Mice with Cre recombinase activatable PDGF-C expression. *Genesis* 32:181-183.
- Eckardt D, Theis M, Degen J, et al. 2004. Functional role of connexin43 gap junction channels in adult mouse heart assessed by inducible gene deletion. *J Mol Cell Cardiol* 36:101-110.
- Eng C, Li F, Abramson D, et al. 1993. Mortality from second tumors among long-term survivors of retinoblastoma. *J Natl Cancer Inst* 85:1121-1128.
- Epifano O, Liang LF, Familiari M, et al. 1995. Coordinate expression of the three zona pellucida genes during mouse oogenesis. *Development* 121:1947-1956.
- Epstein J, Li J, Lang D, et al. 2000. Migration of cardiac neural crest cells in *Spotch* embryos. *Development* 127:1869-1878.
- Fallon J. 1994. FGF-2: Apical ectodermal ridge growth signal for chick limb development. *Science* 264:104-107.
- Feil R, Brocard J, Mascres B, et al. 1996. Ligand-activated site-specific recombination in mice. *Proc Natl Acad Sci USA* 93:10887-10890.
- Feldman B, Poueymirou W, Papaioannou VE, et al. 1995. Requirement of FGF-4 for postimplantation mouse development. *Science* 267:246-249.
- Ferrara N, Carver-Moore K, Chen H, et al. 1996. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 380:439-442.
- Foster JW, Dominguez-Steglich MA, Guioli S, et al. 1994. Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. *Nature* 372:525-530.
- Frengen E, Zhao B, Howe S, et al. 2000. Modular bacterial artificial chromosome vectors for transfer of large inserts into mammalian cells. *Genomics* 68:118-126.
- Friend SH, Bernards R, Rogelj S, et al. 1986. A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature* 323:643-646.
- Fung YK, Murphree AL, T'Ang A, et al. 1987. Structural evidence for the authenticity of the human retinoblastoma gene. *Science* 236:1657-1661.
- Furuta Y, Lagutin O, Hogan BL, et al. 2000. Retina- and ventral forebrain-specific Cre recombinase activity in transgenic mice. *Genesis* 26:130-132.
- Gu Z, Reynolds EM, Song J, et al. 1999. The type I serine/threonine kinase receptor ActRIA (ALK2) is required for gastrulation of the mouse embryo. *Development* 126:2551-2561.
- Guo C, Yang W, Lobe CG. 2002. A Cre recombinase transgene with mosaic, widespread tamoxifen-inducible action. *Genesis* 32:8-18.
- Guo Q, Loomis C, Joyner AL. 2003. Fate map of mouse ventral limb ectoderm and the apical ectodermal ridge. *Dev Biol* 264:166-178.
- Hansen J, Floss T, Van Sloun P, et al. 2003. A large-scale, gene-driven mutagenesis approach for the functional analysis of the mouse genome. *Proc Natl Acad Sci USA* 100:9918-9922.
- Harada N, Tamai Y, Ishikawa T-o, et al. 1999. Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. *EMBO J* 18:5931-5942.
- Hayashi S, McMahon AP. 2002. Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. *Dev Biol* 244:305-318.
- Hayashi M, Kim SW, Imanaka-Yoshida K, et al. 2004. Targeted deletion of BMK1/ERK5 in adult mice perturbs vascular integrity and leads to endothelial failure. *J Clin Invest* 113:1138-1148.
- Hebert JM, McConnell SK. 2000. Targeting of cre to the *Foxg1* (BF-1) Locus Mediates loxP recombination in the telencephalon and other developing head structures. *Dev Biol* 222:296-306.
- Hecht NB, Bower PA, Waters SH, et al. 1986. Evidence for haploid expression of mouse testicular genes. *Exp Cell Res* 164:183-190.
- Heinrich AC, Pelanda R, Klingmuller U. 2004. A mouse model for visualization and conditional mutations in the erythroid lineage. *Blood* 104:659-666.
- Hunter N, Awatramani R, Farley F, et al. 2005. Ligand-activated Flpe for temporally-regulated modifications. *Genesis* 41:99-109.
- Imai T, Jiang M, Chambon P, et al. 2001. Impaired adipogenesis and lipolysis in the mouse upon selective ablation of the retinoid X receptor alpha mediated by a tamoxifen-inducible chimeric Cre recombinase (Cre-ERT2) in adipocytes. *Proc Natl Acad Sci USA* 98:224-228.
- Imai T, Takakuwa R, Marchand S, et al. 2004. Peroxisome proliferator-activated receptor {gamma} is required in mature white and brown adipocytes for their survival in the mouse. *Proc Natl Acad Sci USA* 101:4543-4547.
- Indra AK, Warot X, Brocard J, et al. 1999. Temporally-controlled site-specific mutagenesis in the basal layer of the epidermis: comparison of the recombinase activity of the tamoxifen-inducible Cre-ER(T) and Cre-ER(T2) recombinases. *Nucleic Acids Res* 27:4324-4327.
- Jacks T, Fazeli A, Schmitt EM, et al. 1992. Effects of an Rb mutation in the mouse. *Nature* 359:295-300.
- Jamin SP, Arango NA, Mishina Y, et al. 2002. Requirement of *Bmpr1a* for Mullerian duct regression during male sexual development. *Nat Genet* 32:408-410.
- Justice MJ, Noveroske JK, Weber JS, et al. 1999. Mouse ENU mutagenesis. *Hum Mol Genet* 8:1955-1963.
- Kawamoto S, Niwa H, Tashiro F, et al. 2000. A novel reporter mouse strain that expresses enhanced green fluorescent protein upon Cre-mediated recombination. *FEBS Lett* 470:263-268.
- Kellendonk C, Tronche F, Monaghan A-P, et al. 1996. Regulation of Cre recombinase activity by the synthetic steroid RU 486. *Nucleic Acids Res* 24:1404-1411.
- Kimmel RA, Turnbull DH, Blanquet V, et al. 2000. Two lineage boundaries coordinate vertebrate apical ectodermal ridge formation. *Genes Dev* 14:1377-1389.
- Kist R, Schrewe H, Balling R, et al. 2002. Conditional inactivation of *Sox9*: a

- mouse model for campomelic dysplasia. *Genesis* 32:121-123.
- Kuhn R, Schwenk F, Aguet M, et al. 1995. Inducible gene targeting in mice. *Science* 269:1427-1429.
- Le DT, Kong N, Zhu Y, et al. 2004. Somatic inactivation of Nf1 in hematopoietic cells results in a progressive myeloproliferative disorder. *Blood* 103:4243-4250.
- Lee WH, Bookstein R, Hong F, et al. 1987. Human retinoblastoma susceptibility gene: cloning, identification, and sequence. *Science* 235:1394-1399.
- Lee EY, Hu N, Yuan SS, et al. 1994. Dual roles of the retinoblastoma protein in cell cycle regulation and neuron differentiation. *Genes Dev* 8:2008-2021.
- Lee M, Williams B, Mulligan G, et al. 1996. Targeted disruption of p107: functional overlap between p107 and Rb. *Genes Dev* 10:1621-1632.
- Lewandoski M, Martin GR. 1997. Cre-mediated chromosome loss in mice. *Nat Genet* 17:223-225.
- Lewandoski M, Wassarman KM, Martin GR. 1997. Zp3-cre, a transgenic mouse line for the activation or inactivation of loxP-flanked target genes specifically in the female germ line. *Curr Biol* 7:148-151.
- Lewandoski M, Sun X, Martin GR. 2000. Fgf8 signalling from the AER is essential for normal limb development. *Nat Genet* 26:460-463.
- Li M, Indra AK, Warot X, et al. 2000. Skin abnormalities generated by temporally controlled RXR α mutations in mouse epidermis. *Nature* 407:633-636.
- Liem KF Jr, Tremml G, Jessell TM. 1997. A role for the roof plate and its resident TGF β -related proteins in neuronal patterning in the dorsal spinal cord. *Cell* 91:127-138.
- Lobe CG, Koop KE, Kreppner W, et al. 1999. Z/AP, a double reporter for Cre-mediated recombination. *Dev Biol* 208:281-292.
- Logie C, Stewart AF. 1995. Ligand-regulated site-specific recombination. *Proc Natl Acad Sci USA* 92:5940-5944.
- Maandag E, van der Valk M, Vlaar M, et al. 1994. Developmental rescue of an embryonic-lethal mutation in the retinoblastoma gene in chimeric mice. *EMBO J* 13:4260-4268.
- MacPherson D, Sage J, Kim T, et al. 2004. Cell type-specific effects of Rb deletion in the murine retina. *Genes Dev* 18:1681-1694.
- Mao X, Fujiwara Y, Orkin SH. 1999. Improved reporter strain for monitoring Cre recombinase-mediated DNA excisions in mice. *Proc Natl Acad Sci USA* 96:5037-5042.
- Mao X, Fujiwara Y, Chapdelaine A, et al. 2001. Activation of EGFP expression by Cre-mediated excision in a new ROSA26 reporter mouse strain. *Blood* 97:324-326.
- McLean GW, Komiyama NH, Serrels B, et al. 2004. Specific deletion of focal adhesion kinase suppresses tumor formation and blocks malignant progression. *Genes Dev* 18:2998-3003.
- Metzger D, Clifford J, Chiba H, et al. 1995. Conditional site-specific recombination in mammalian cells using a ligand-dependent chimeric Cre recombinase. *Proc Natl Acad Sci USA* 92:6991-6995.
- Mishina Y, Suzuki A, Ueno N, et al. 1995. Bmpr encodes a type I bone morphogenetic protein receptor that is essential for gastrulation during mouse embryogenesis. *Genes Dev* 9:3027-3037.
- Mishina Y, Crombie R, Bradley A, et al. 1999. Multiple roles for activin-like kinase-2 signaling during mouse embryogenesis. *Dev Biol* 213:314-326.
- Mishina Y, Hanks MC, Miura S, et al. 2002. Generation of Bmpr/Alk3 conditional knockout mice. *Genesis* 32:69-72.
- Mishina Y. 2003. Function of bone morphogenetic protein signaling during mouse development. *Front Biosci* 1:d855-d869.
- Moon AM, Capecchi MR. 2000. Fgf8 is required for outgrowth and patterning of the limbs. *Nat Genet* 26:455-459.
- Moon AM, Boulet AM, Capecchi MR. 2000. Normal limb development in conditional mutants of Fgf4. *Development* 127:989-996.
- Moses KA, DeMayo F, Braun RM, et al. 2001. Embryonic expression of an Nkx2-5/Cre gene using ROSA26 reporter mice. *Genesis* 31:176-180.
- Motoike T, Markham DW, Rossant J, et al. 2003. Evidence for novel fate of Flk1+ progenitor: contribution to muscle lineage. *Genesis* 35:153-159.
- Murali D, Yoshikawa S, Corrigan RR, et al. 2005. Distinct developmental programs require different levels of Bmp signaling during mouse retinal development. *Development* 132:913-923.
- Nagy A. 2000. Cre recombinase: the universal reagent for genome tailoring. *Genesis* 26:99-109.
- Ng JK, Tamura K, Buscher D, et al. 1999. Molecular and cellular basis of pattern formation during vertebrate limb development. *Curr Top Dev Biol* 41:37-66.
- Niswander L, Tickle C, Vogel A, et al. 1993. FGF-4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb. *Cell* 75:579-587.
- Niwa H, Yamamura K, Miyazaki J. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108:193-200.
- Novak A, Guo C, Yang W, et al. 2000. Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. *Genesis* 28:147-155.
- O'Gorman S, Dagenais NA, Qian M, et al. 1997. Protamine-Cre recombinase transgenes efficiently recombine target sequences in the male germ line of mice, but not in embryonic stem cells. *Proc Natl Acad Sci USA* 94:14602-14607.
- Palmiter RD, Brinster RL. 1986. Germ-line transformation of mice. *Annu Rev Genet* 20:465-499.
- Panchision DM, Pickel JM, Studer L, et al. 2001. Sequential actions of BMP receptors control neural precursor cell production and fate. *Genes Dev* 15:2094-2110.
- Peschon JJ, Behringer RR, Brinster RL, et al. 1987. Spermatid-specific expression of protamine 1 in transgenic mice. *Proc Natl Acad Sci USA* 84:5316-5319.
- Pizette S, Abate-Shen C, Niswander L. 2001. BMP controls proximodistal outgrowth, via induction of the apical ectodermal ridge, and dorsoventral patterning in the vertebrate limb. *Development* 128:4463-4474.
- Sadek S, Bell SC. 1996. The effects of the antihormones RU486 and tamoxifen on fetoplacental development and placental bed vascularisation in the rat: a model for intrauterine fetal growth retardation. *Br J Obstet Gynaecol* 103:630-641.
- Salim A, Wiknjosastro GH, Danukusumo D, et al. 1998. Fetal retinoblastoma. *J Ultrasound Med* 17:717-720.
- Schmidt EE, Taylor DS, Prigge JR, et al. 2000. Illegitimate Cre-dependent chromosome rearrangements in transgenic mouse spermatids. *Proc Natl Acad Sci* 97:13702-13707.
- Schneider A, Guan Y, Zhang Y, et al. 2004. Generation of a conditional allele of the mouse prostaglandin EP4 receptor. *Genesis* 40:7-14.
- Schwenk F, Kuhn R, Angrand P-O, et al. 1998. Temporally and spatially regulated somatic mutagenesis in mice. *Nucleic Acids Res* 26:1427-1432.
- Seibler J, Zevnik B, Kuter-Luks B, et al. 2003. Rapid generation of inducible mouse mutants. *Nucleic Acids Res* 31:e12.
- Sgaier SK, Millet S, Villanueva MP, et al. 2005. Morphogenetic and cellular movements that shape the mouse cerebellum: insights from genetic fate mapping. *Neuron* 45:27-40.
- Soriano P. 1999. Generalized lacZ expression with ROSA26 Cre reporter strain. *Nat Genet* 21:70-71.
- Soshnikova N, Zechner D, Huelsken J, et al. 2003. Genetic interaction between Wnt/ β -catenin and BMP receptor signaling during formation of the AER and the dorsal-ventral axis in the limb. *Genes Dev* 17:1963-1968.
- Srinivas S, Watanabe T, Lin CS, et al. 2001. Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Dev Biol* 1:27.
- Stryke D, Kawamoto M, Huang CC, et al. 2003. BayGenomics: a resource of insertional mutations in mouse embryonic stem cells. *Nucleic Acids Res* 31:278-281.
- Sun X, Meyers EN, Lewandoski M, et al. 1999. Targeted disruption of Fgf8 causes failure of cell migration in the gastrulating mouse embryo. *Genes Dev* 13:1834-1846.

- Sun X, Lewandoski M, Meyers EN, et al. 2000. Conditional inactivation of *Fgf4* reveals complexity of signalling during limb bud development. *Nat Genet* 25:83-86.
- Sun X, Mariani FV, Martin GR. 2002. Functions of FGF signalling from the apical ectodermal ridge in limb development. *Nature* 418:501-508.
- Tao W, Lai E. 1992. Telencephalon-restricted expression of BF-1, a new member of the HNF-3/fork head gene family, in the developing rat brain. *Neuron* 8:957-966.
- Tian H, Jeong J, Harfe BD, et al. 2005. Mouse *Disp1* is required in sonic hedgehog-expressing cells for paracrine activity of the cholesterol-modified ligand. *Development* 132:133-142.
- van Rijen HV, Eckardt D, Degen J, et al. 2004. Slow conduction and enhanced anisotropy increase the propensity for ventricular tachyarrhythmias in adult mice with induced deletion of *connexin43*. *Circulation* 109:1048-1055.
- Vasioukhin V, Degenstein L, Wise B, et al. 1999. The magical touch: genome targeting in epidermal stem cells induced by tamoxifen application to mouse skin. *Proc Natl Acad Sci USA* 96:8551-8556.
- Vintersten K, Monetti C, Gertsenstein M, et al. 2004. Mouse in red: red fluorescent protein expression in mouse ES cells, embryos, and adult animals. *Genesis* 40:241-246.
- Vooijs M, Jonkers J, Berns A. 2001. A highly efficient ligand-regulated Cre recombinase mouse line shows that *LoxP* recombination is position dependent. *EMBO Rep* 2:292-297.
- Vooijs M, te Riele H, van der Valk M, et al. 2002. Tumor formation in mice with somatic inactivation of the retinoblastoma gene in interphotoreceptor retinol binding protein-expressing cells. *Oncogene* 21:4635-4645.
- Wagner T, Wirth J, Meyer J, et al. 1994. Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene *SOX9*. *Cell* 79:1111-1120.
- Wells CM, Walmsley M, Ooi S, et al. 2004. *Rac1*-deficient macrophages exhibit defects in cell spreading and membrane ruffling but not migration. *J Cell Sci* 117:1259-1268.
- Williams B, Schmitt E, Remington L, et al. 1994. Extensive contribution of *Rb*-deficient cells to adult chimeric mice with limited histopathological consequences. *EMBO J* 13:4251-4259.
- Wine-Lee L, Ahn KJ, Richardson RD, et al. 2004. Signaling through BMP type 1 receptors is required for development of interneuron cell types in the dorsal spinal cord. *Development* 131:5393-5403.
- Wolpert L. 2002. The progress zone model for specifying positional information. *Int J Dev Biol* 46:869-870.
- Wu L, de Bruin A, Saavedra HI, et al. 2003. Extra-embryonic function of *Rb* is essential for embryonic development and viability. *Nature* 421:942-947.
- Wunderlich FT, Wildner H, Rajewsky K, et al. 2001. New variants of inducible Cre recombinase: a novel mutant of Cre-PR fusion protein exhibits enhanced sensitivity and an expanded range of inducibility. *Nucleic Acids Res* 29:E47.
- Xu J, Liu Z, Ornitz DM. 2000. Temporal and spatial gradients of *Fgf8* and *Fgf17* regulate proliferation and differentiation of midline cerebellar structures. *Development* 127:1833-1843.
- Xu H, Morishima M, Wylie JN, et al. 2004. *Tbx1* has a dual role in the morphogenesis of the cardiac outflow tract. *Development* 131:3217-3227.
- Yi SE, Daluiski A, Pederson R, et al. 2000. The type I BMP receptor *BMPRII* is required for chondrogenesis in the mouse limb. *Development* 127:621-630.
- Zhang X, Heaney S, Maas RL. 2003. Cre-loxP fate-mapping of *Pax6* enhancer active retinal and pancreatic progenitors. *Genesis* 35:22-30.
- Zhang J, Gray J, Wu L, et al. 2004. *Rb* regulates proliferation and rod photoreceptor development in the mouse retina. *Nat Genet* 36:351-360.
- Zhao GQ. 2003. Consequences of knocking out BMP signaling in the mouse. *Genesis* 35:43-56.
- Zhou Z, Wang D, Wang XJ, et al. 2002. In utero activation of *K5.CrePR1* induces gene deletion. *Genesis* 32:191-192.
- Zinyk D, Mercer EH, Harris E, et al. 1998. Fate mapping of the mouse midbrain-hindbrain constriction using a site-specific recombination system. *Curr Biol* 8:665-668.
- Zirlinger M, Lo L, McMahon J, et al. 2002. Transient expression of the bHLH factor neurogenin-2 marks a subpopulation of neural crest cells biased for a sensory but not a neuronal fate. *Proc Natl Acad Sci USA* 99:8084-8089.

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☒ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.